A Preclinical Model of Heterogeneous Traumatic Brain Injury

A Thesis Presented to The Academic Faculty

By

Kyle Sean Milligan

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in Bioengineering

Georgia Institute of Technology

August 2018

A Preclinical Model of Heterogeneous Traumatic Brain Injury

Approved by:

Dr. Michelle LaPlaca, Advisor
Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Erin Buckley
Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Christopher Rozell
School of Electrical and Computer Engineering
Georgia Institute of Technology

Date Approved: July 27 2018

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge the researchers and student scientists who contributed to this work – our lab technicians, undergraduate research assistants, and my fellow graduate students: Eric Gaupp, Makeeva Walker, David Namkoong, Caroline Ware, Andrew Bassett, Connor Sofia, Delaney Beckner, Michael Assan, Tarun Maddali, Erisa Sula, Anvesh Chennuru, Srujana Buddi, and Scott Hogan. I would also like to thank our frequent collaborators at Emory University: Dr. Erin Buckley and the members of her lab, especially Bharat Sanders, who contributed their knowledge, equipment, and time to the biomechanics portion of this study. I am grateful to the National Institutes of Health for funding this work.

I am fortunate to have been surrounded by knowledgeable and generous professors and advisors at Georgia Tech. My faculty advisor, Dr. Michelle LaPlaca, has been an invaluable resource and a supportive mentor, and I am thankful for her guidance, kindness, and enthusiasm throughout this project. I would like to thank the other members of my faculty committee – Dr. Christopher Rozell and Dr. Erin Buckley - for their time and candid feedback. My academic advisor, Laura

Paige, has also been an endless source of help and encouragement.

I am grateful to my friends, who have encouraged and supported me throughout this program, and to my teachers, for enabling me to get here. Finally, I am grateful to my family: Austin, Matthew, Mom, and Dad – I love you guys. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

CCI	Controlled cortical impact
EVA	Ethylene-vinyl acetate
FPI	Fluid percussion injury
GFAP	Glial fibrillary acidic protein
NOR	Novel object recognition
PU	Polyurethane
TBI	Traumatic brain injury

SUMMARY

Traumatic brain injury (TBI) is a leading cause of death and disability in the United States, but the factors affecting clinical outcomes following TBI are complex. Animal TBI models are widely used, but many design parameters go largely unreported. We evaluate the effects of one such parameter, head support foam type, on injury outcome in rats. We hypothesized that TBI severity is increased on stiffer foams.

TBI was delivered to the closed head of 54 rats using a controlled cortical impact (CCI) device. We analyzed injury biomechanics on four foams using an accelerometer and high-speed video, and performed histopathology to evaluate tissue response. Our results indicate that foam type can significantly affect injury biomechanics and cellular outcomes, but the mechanical properties of the foam were not predictive of outcome. We recommend more consistent reporting of foam type and origin, and suggest that a full mechanical characterization of individual foam choices is likely unnecessary. We also introduce an experiment to study the effects of population heterogeneity on TBI outcomes, and discuss the use of common data elements (CDEs) as a reporting tool.

CHAPTER 1

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disability in the United States (Taylor et al. 2017). Despite its high incidence, a robust understanding of the mechanisms and outcomes of brain injury has proved elusive. This may be due to a number of factors, including the complexity of TBI and the difficulties of studying it in humans. No two injuries are the same, and symptoms may vary depending on the severity and location of impact.

This heterogeneity is especially evident in the case of mild TBI (mTBI), where the lesser severity of injury may decrease the signal-to-noise ratio; symptoms could be present immediately, may not develop until days or weeks after injury, or may go unrecognized entirely (NIH 2002). Clinical diagnosis is rendered even more difficult by the fact that many patients have one or more complicating factors, such as alcohol consumption or drug use near the time of hospital admission (Furger et al. 2015). Furthermore, a standardized definition of clinical mild TBI (mTBI) has not been firmly established; in a 2004 review, the World Health Organization found that the heterogeneity in case definitions of mTBI has

had a negative impact on the interpretation and comparison of research findings (Carroll et al. 2004).

These difficulties necessitate the use of animal surrogates including mice and rats. Individual animal models are typically designed to produce homogeneous injuries, with demographic features (sex, age, etc.) and injury parameters tightly controlled. This imposed homogeneity prevents any one model from reflecting the heterogeneous nature of the clinical disease. Across the preclinical literature, however, there is a wide array of heterogeneous methods and outcome metrics (Xiong et al. 2013).

There are several common preclinical injury models, many of which are not fully characterized and so have limited replicability. For example, a number of methods involve impacting the animal's head on a block of foam or other material (Marmarou et al. 1994, Prins et al. 2010, Petraglia et al. 2014, Jamnia et al. 2016), the material properties of which may influence injury response but are rarely reported. This additional level of unintended heterogeneity, due to differences in injury model design and characterization, may contribute to the reported difficulties in comparing and replicating preclinical studies (Smith et al. 2015).

We hypothesize that these under-reported parameters of model design play a significant role in TBI response. In this

study, we evaluate the effects of modulating one such parameter, head support foam type, in a closed-head controlled cortical impact model of mTBI in rats. We aimed to determine whether the material properties of the foam used to cushion the head during injury affect either the biomechanics of the injury or the post-injury tissue response.

CHAPTER 2

LITERATURE REVIEW

A number of methods to induce brain injury are reported in the preclinical TBI literature. The most common include controlled cortical impact (CCI) injury, weight drop injury, fluid percussion injury (FPI), and blast injury. Each of these models has multiple variations, including whether the skull was open or closed during injury, whether the head is fixed in place or allowed to move freely, and whether the injury was given laterally or centrally (Xiong et al. 2013).

The controlled cortical impact (CCI) device was introduced by Lighthall in the Journal of Neurotrauma in 1988. CCI injuries are delivered by a pneumatic piston driven at a specified depth and velocity to contact the head. This early experimental model was used to induce open-head TBI in ferrets, with impact velocities ranging between 2-4m/s and displacement of 2-5mm in a fixed head. Reported were pathophysiological results ranging from no apparent systemic changes using the least severe injury parameters (2m/s, 2mm) to immediate fatality using the most severe (4m/s, 4mm) (Lighthall 1988). The benefits of the CCI model are that the impact velocity, duration, and depth are all controlled to a degree not feasible in other models. Later versions of this

model adapted the CCI device for a closed-head injury, with the head placed on a block of foam or wood during impact (Prins et al. 2010, Petraglia et al. 2014, Jamnia et al. 2016).

The weight drop model, introduced by Marmarou et al. in 1994 is also frequently used, perhaps because of its straightforward design and simple implementation. The weight drop model consists of a plastic guide tube and a weight that is dropped through the tube onto the head. The original Marmarou model used a 450 gram weight dropped from a height of 2m to induce a diffuse moderate injury. In this model, the head was placed on a bed made of an uncharacterized foam purchased from a small local supplier (Foam to Size, Inc., Ashland VA) (Marmarou et al. 1994). Weight drop models are simpler and less costly to implement than CCI, and can generate greater rotational accelerations than the linear CCI injury. Actual rotational accelerations depend on the particular experimental setup, including height and weight of the falling object (Chen et al. 1996, Feeney et al. 1981, Flierl et al. 2009, Kilbourne et al. 2009).

Fluid percussion injury (FPI) models use a pendulum to suddenly force water onto the intact dura through a tubular reservoir (Galgano et al. 2015). This model produces brief deformation of the brain tissue, with exact severity

depending on the strength of the pressure pulse. Pulse strength is modulated by the initial height of a falling pendulum, which strikes a piston at one end of the fluid tube. This model offers less control over the biomechanics of injury, as the only adjustable mechanical parameter is the initial pendulum height (McIntosh et al. 1987, Carbonell et al. 1998, Bramlett et al. 1999).

Blast injury models are also frequently used, particularly in the study of military TBI, but deliver injury without impacting the head or brain directly. Blast models direct a concussive blast wave, driven by compressed gas, to the head or exposed brain (Long et al. 2009, Cheng et al. 2010, Reneer et al. 2011).

In addition to the variety of injuries induced by these different TBI models, there are a number of factors that vary even between different applications of the same model. In CCI, for instance, impact can be delivered to the open skull in a fixed head (Lighthall 1988), or to a closed, unfixed head on a foam bed (Jamnia et al. 2016) or on a wooden block (Prins et al. 2010). In weight drop models, the head can be placed on a foam bed as in the original Marmarou 1994 study, or on scored tin foil that allows completely free movement of the head following injury (Mychasiuk et al. 2014). Additional complication is provided by the type of anesthesia

(Wojnarowicz et al. 2017) and the particular species of animal used; results of a TBI model from one species may not match those from another (Johnson et al. 2015). While the diversity of models may more closely approximate the heterogeneous nature of clinical TBI, small modifications to an injury model can have dramatic effects on outcome (Smith et al. 2015).

Some recent work suggests that heterogeneity may, in fact, improve reproducibility. In an analysis of single- versus multi-laboratory studies across 13 different interventions in preclinical models of stroke, breast cancer, and myocardial infarction, multi-laboratory studies predicted effect size up to 42% more accurately. These results were attributed in part to over-standardization in animal research, to the point that results may be more reflective of differences between laboratories and animal phenotypes than of genuine scientific findings (Voelkl and Würbel 2016, Voelkl et al. 2018). These results indicate that thoughtful standardization of some model parameters and careful implementation of deliberate heterogeneity may improve reproducibility and validity of preclinical results.

In addition to injury administration, preclinical models have also struggled to translate the variety of clinical outcomes to animals. Depending on the location and severity of the injury, damage may range from mild to moderate to

severe, and may affect different brain structures. Cortical damage is frequently cited, but the cognitive deficits often found in behavioral assays after TBI are also indicative of hippocampal damage (Hamm et al. 1992). These assays include novel object recognition (NOR), in which the animal is presented with one set of objects for a period of time, followed by a second set in which one of the original objects has been replaced by a novel object. The time spent exploring each object is recorded; a neurotypical animal should spend more time exploring the novel object than the familiar one. NOR has been successfully used in a number of TBI studies to gauge cognitive deficits (Siopi et al. 2012, Rachmany et al. 2013, Grayson et al. 2015).

Beyond behavioral outcomes, histopathology is a standard method to evaluate tissue response following preclinical TBI. A number of proteins are expressed in the injured brain, including the Alzheimer's-linked amyloid-β (Johnson et al. 2010), but a frequently used marker of tissue damage is glial fibrillary acidic protein (GFAP), expressed by astrocytes in response to injury (Papa et al. 2014, Cikriklar et al. 2016, Zhang et al. 2016).

CHAPTER 3

METHODS

3.1 Experimental design

This experiment is the first part of a broader study aimed at understanding preclinical TBI heterogeneity. That is, understanding how various demographic and experimental variables affect TBI outcomes. This experiment was designed to address certain model parameters that may influence the severity of injury in a preclinical CCI model, and that often go unreported in literature.

In particular, we examined the effects of using different types of foam to support the head during injury. This is an element of model design that is not standardized or commonly reported, but one that may be important in characterizing an injury model. The second experiment in the study is introduced in Chapter 6, and is aimed at understanding the role of demographic variables, like sex and strain, in preclinical injury outcome. We also describe our contribution to a standardized reporting method using Common Data Elements (CDEs).

3.2 Procedures

3.2.1 Animals

A total of 41 adult (11-13 weeks old) Sprague Dawley rats, 25 male and 16 female, were acquired from Charles River. The male rats were divided into four experimental groups and one sham group (Table 3.1), and the females into two experimental groups (n=6 or 10). To reduce the number of animals used, the females in the smaller group each underwent four injuries, one on each foam. All rats were double-housed for a minimum of five days prior to any procedures, with free access to food and water.

Table 3.1 Animal group assignments. (*) indicates the same 6 animals were used to test different foams

Sex	EVA	Marmarou	PU	Lab	Sham
Male	7	2	4	7	4
Female	10, 3 m/s 6*, 5 m/s	6*	6*	6*	--

3.2.2 Foam selection

Four different foams were used to support the head during injury. The foams were selected based on their material properties and any prior use in literature. Closed-cell ethylene-vinyl acetate (EVA) and open-cell polyurethane (PU) foam were acquired from McMaster-Carr. The foam used in the original 1994 Marmarou weight-drop experiment was obtained

from Foam to Size, Inc. (Ashland, VA). A fourth, UV-damaged foam was selected from existing laboratory supplies.

Foam density was calculated from the mass and volume of a 2-inch cube of each foam type (density = mass/volume). Foam cell structure (open/closed) was determined from microscopy. Young's modulus, the ratio of stress to strain used as a measure of stiffness in solid materials, was determined for each foam by compression testing following ASTM standards using an MTS 858 Mini Bionix II. Foam properties are reported in Table 3.2 and representative images showing foam cell structure are presented in Figure 3.1.

Table 3.2 Foam material properties

Foam	EVA	Marmarou	PU	Lab
Density (g/cm^3)	0.046	0.017	0.036	0.031
Young's modulus (KPa)	106.11	12.33	74.78	6.04
Cell structure	Closed	Open	Open	Open

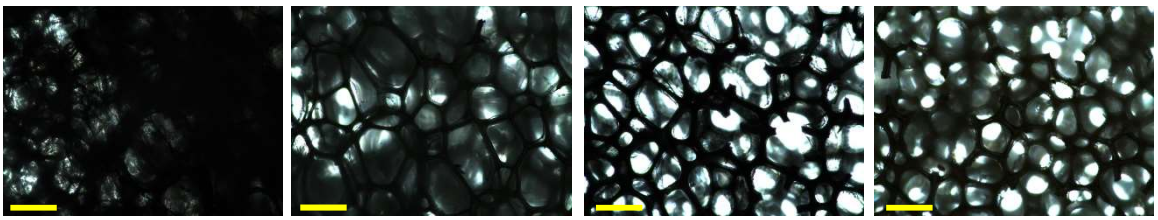

Figure 3.1 Cell structure of foams was determined by microscopy. Left to right: EVA, Marmarou, PU, lab foam. Scale bars = 0.5 mm.

3.2.3 High-speed video & accelerometer tracking

Head motion was tracked using a high-speed camera (Sony RX100 IV, 980 FPS) and a ruler placed adjacent to the head. The acceleration of the head during impact was recorded using an Endevco model 25A Isomin accelerometer affixed in the mouth behind the lower front teeth, sampling at a rate of 20 kHz.

3.2.4 TBI induction

Animals were anesthetized with isoflurane (3-5% induction, 1.5-3% maintenance) and weighed. Ketoprofen (5mg/kg) was then administered by intradermal injection. Anesthesia duration was monitored and recorded. The head was placed on a block of foam underneath the impactor, with the body resting level with the head. The foam block was 2 inches tall and 1 inch wide. A transparent ruler was placed adjacent to the head to track displacement using the camera.

Injury was delivered using controlled cortical impact (CCI) (Pittsburgh Precision Instruments) to the intact skull, posterior and adjacent to bregma along the sagittal suture, in an unfixed head. Six of the females placed on the EVA foam received a higher velocity impact than the rest of the animals (5m/s, 5mm displacement, 50ms contact duration). All other animals received a less severe injury (3m/s, 5mm displacement, 100ms contact duration). The impactor was

fitted with a 1.6cm diameter silicone tip (Yield House Industries). Sham animals underwent all steps except for impact (n=8). Animals were immediately moved to a heated pad following injury, to assist recovery from anesthesia.

3.2.5 Acute neurological response

The time from injury to recovery of the toe pinch response, and the total righting time, were recorded using a stopwatch.

3.2.6 Animal sacrifice

Animals were sacrificed 24 hours after the procedure. Animals were anesthetized with isoflurane (3-5%), and a mixture of ketamine/xylazine/acepromazine (50/10/1.67 mg/kg) was administered by intradermal injection. Animals were then perfused intracardially with a fixative solution of isotonic phosphate buffered saline (PBS) followed by 4% paraform-aldehyde in isotonic phosphate buffer. Following perfusion, brains were removed from the skull and prepared for histological procedures.

3.2.7 Histology

Frozen tissue sections were cut at a thickness of 20µm up to 1mm on either side of the center of the impact site using a Microm HM 550 cryostat (Thermo Fisher Scientific). Sectioned samples were mounted and rabbit polyclonal antibody against GFAP (Dako) was used to stain the brain sections (1:1000 primary, 1:500 secondary dilution).

3.2.8 Imaging & cell counting

Representative images were taken of male cortices near the location of injury using a fluorescence microscope (Nikon Eclipse 80i), in a blinded fashion. Three sections were chosen from each brain such that the sections spanned the injury site from anterior to posterior. Three images were then taken of the cortex in each of these sections, one from near the midline and one each from the left and right hemispheres, for a total of nine images per brain. Image processing and cell counting were performed in ImageJ (NIH) to quantify the degree of GFAP expression based on pixel intensity. Further data analysis was performed in MATLAB.

CHAPTER 4

RESULTS

4.1 Injury biomechanics

4.1.1 High speed video

The total range of motion of the head was calculated for each animal and foam based on frame-by-frame video analysis. Ethylene-vinyl-acetate and polyurethane foam both permitted significantly less total motion than either the Marmarou or lab foam ($p < 0.05$), and EVA foam allowed less movement than PU foam. There was no significant difference in range of motion between Marmarou and Lab foams. Animals injured on the Marmarou and lab foams exhibited a range of motion much greater than the 10mm total prescribed by the injury device (Figure 4.1). Total range of motion was predicted from foam density according to the formula $y = -1013x + 55.4$ ($R^2 = 0.6619$) (Figure 4.2), and from Young's modulus according to the formula $y = -0.296x + 37.4$ ($R^2 = 0.8815$) (Figure 4.3).

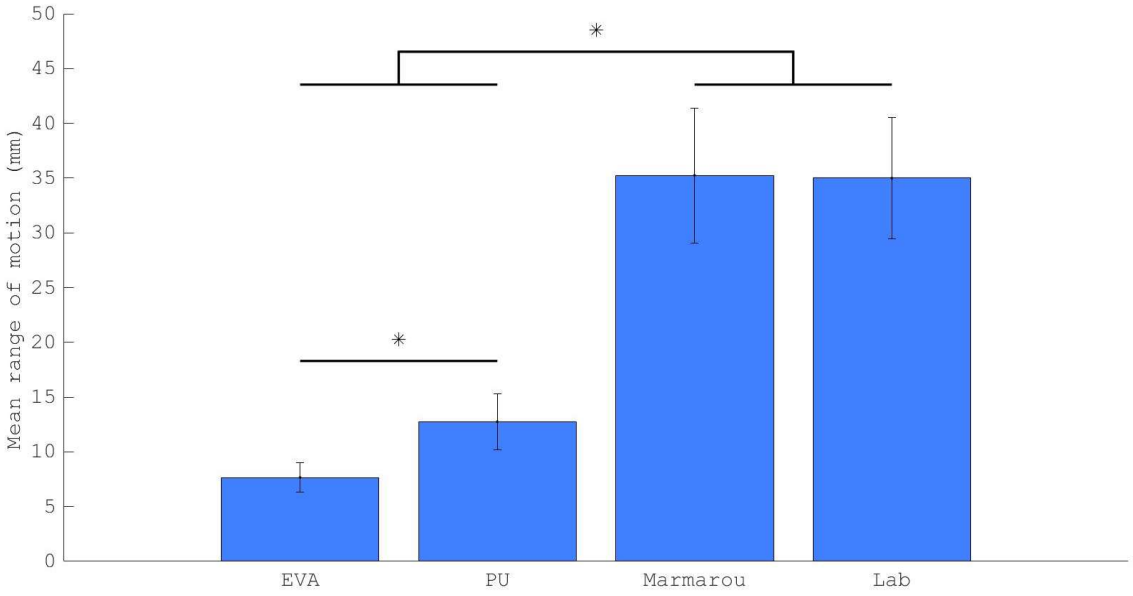

Figure 4.1 Mean total range of motion of the head

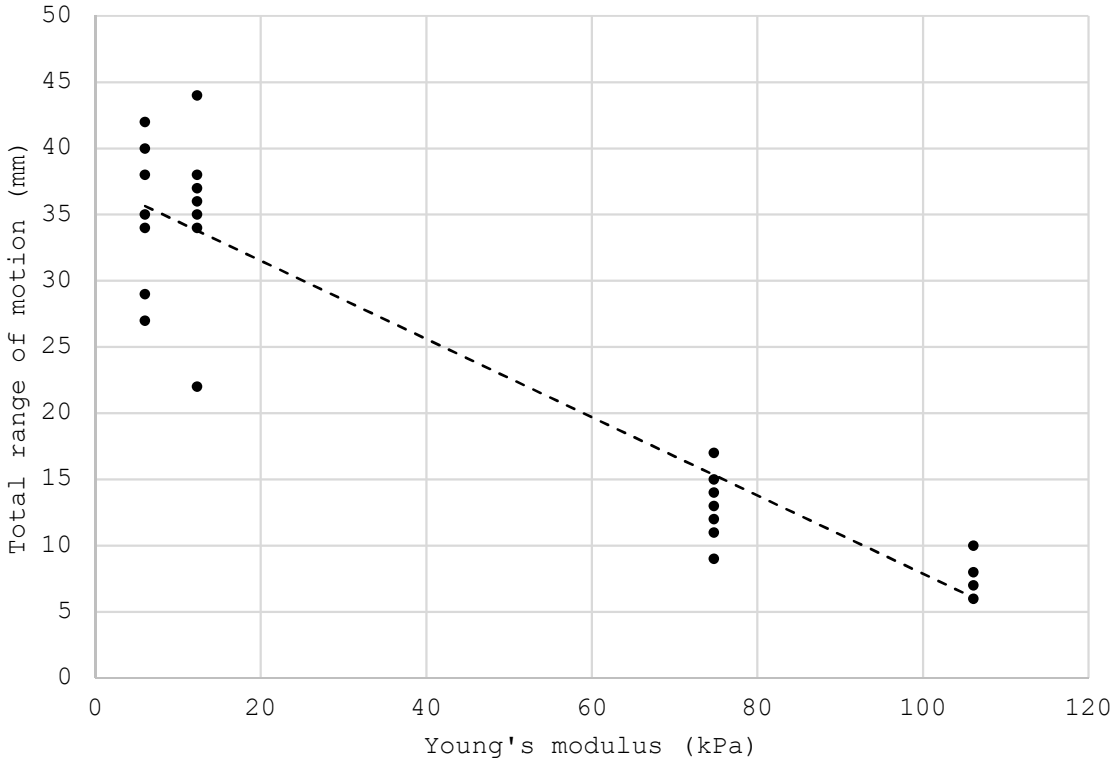

Figure 4.2 Young's modulus predicts range of motion of the head, $R^2 = 0.8815$

Rebounding hits were observed in animals injured on every type of foam. These "second hits" were caused by the rebounding head hitting the impactor for a second time before the impactor had retracted to the up position. Rebounds were more frequently observed on Marmarou and Lab foam than on PU or EVA, but these differences did not reach statistical significance ($p > 0.05$)(Figure 4.3). However, rebound frequency was predicted from Young's Modulus according to the formula $y = -0.0037x + 0.7142$, $R^2 = 0.9268$ (Figure 4.4).

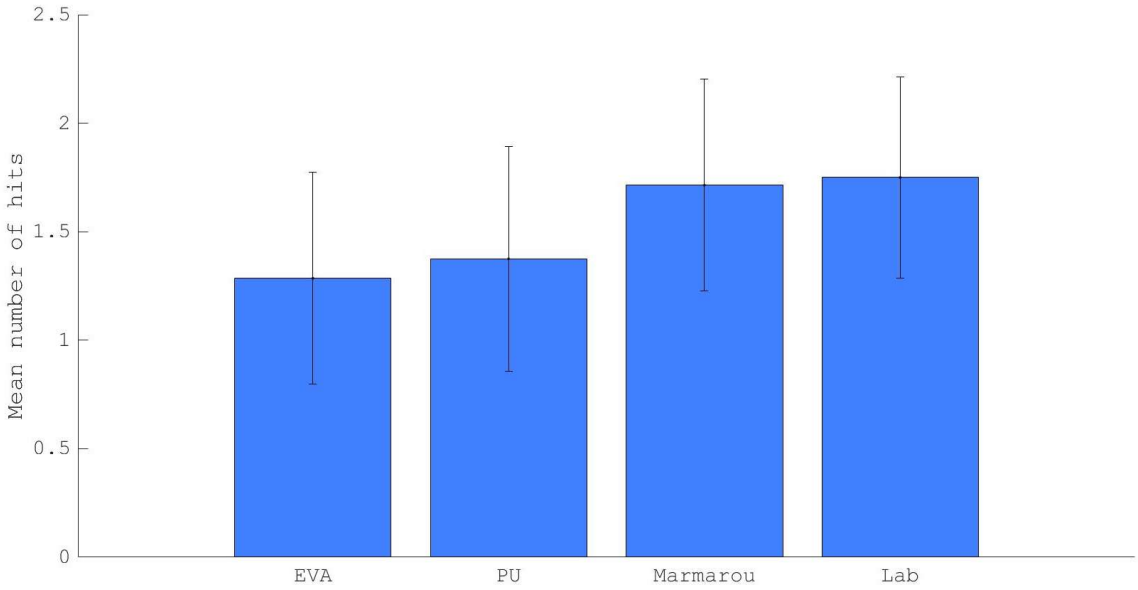

Figure 4.3 Mean number of hits per administered impact on each foam. Foam effects on rebound hits did not reach statistical significance.

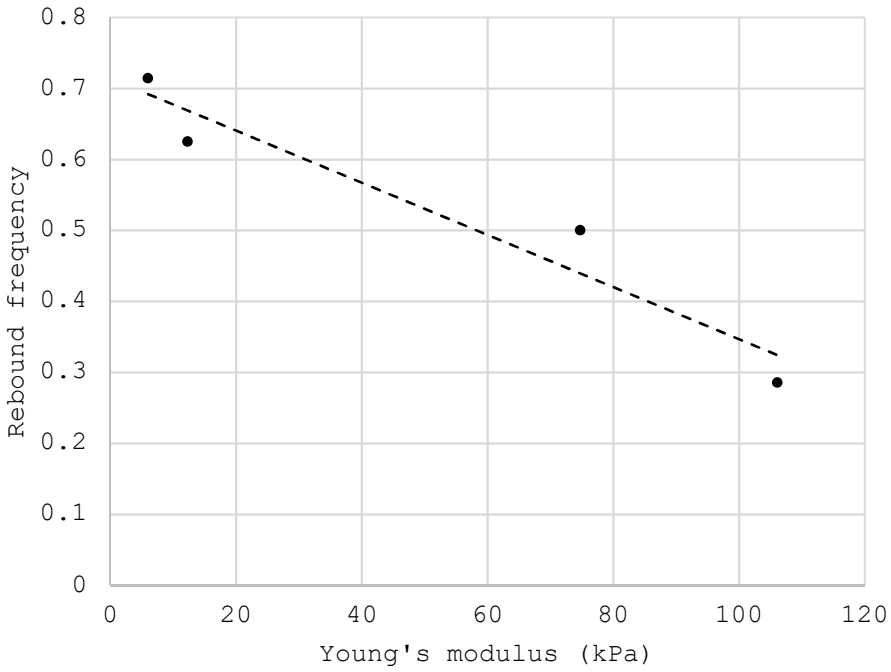

Figure 4.4 Young's modulus predicts head rebound frequency, $R^2 = 0.9268$

4.1.2 Accelerometer results

Acceleration of the head was recorded at 20kHz for the female rats during injury on each type of foam. Acceleration was integrated to find velocity, and integrated a second time to recover displacement. Peak velocity and peak acceleration were calculated for each animal and for each group of animals from the same foam. Average peak accelerations and velocities for each foam are given in Table 4.1.

Table 4.1 Average peak head velocity and acceleration

	Imposed Velocity (m/s)	Peak Velocity (m/s)	Peak Acceleration (m/s^2)
EVA	5	3.44	6182
EVA	3	2.50	6431
PU	3	2.72	5541
Marmarou	3	2.58	4621
Lab	3	2.87	5416

Average peak head acceleration was not significantly different between impacts administered at 3m/s on EVA, Marmarou, PU, or Lab foams. The average peak head acceleration of animals injured at 5m/s on EVA foam was significantly higher than the 3m/s injuries on all foams except EVA. There was no significant difference in peak acceleration between the 3m/s injuries and the 5m/s injuries on EVA foam (Figure 4.5). Average peak velocity was not significantly different between any of the foams at 3m/s, but the 5m/s injury on EVA foam reached a higher peak velocity than all the 3m/s injuries (Figure 4.6). Linear regression was performed on pairs of variables; R^2 values are shown in Table 4.2. Neither Young's modulus nor foam density were highly predictive of velocity or acceleration, but all R^2 values were higher for density than for Young's modulus.

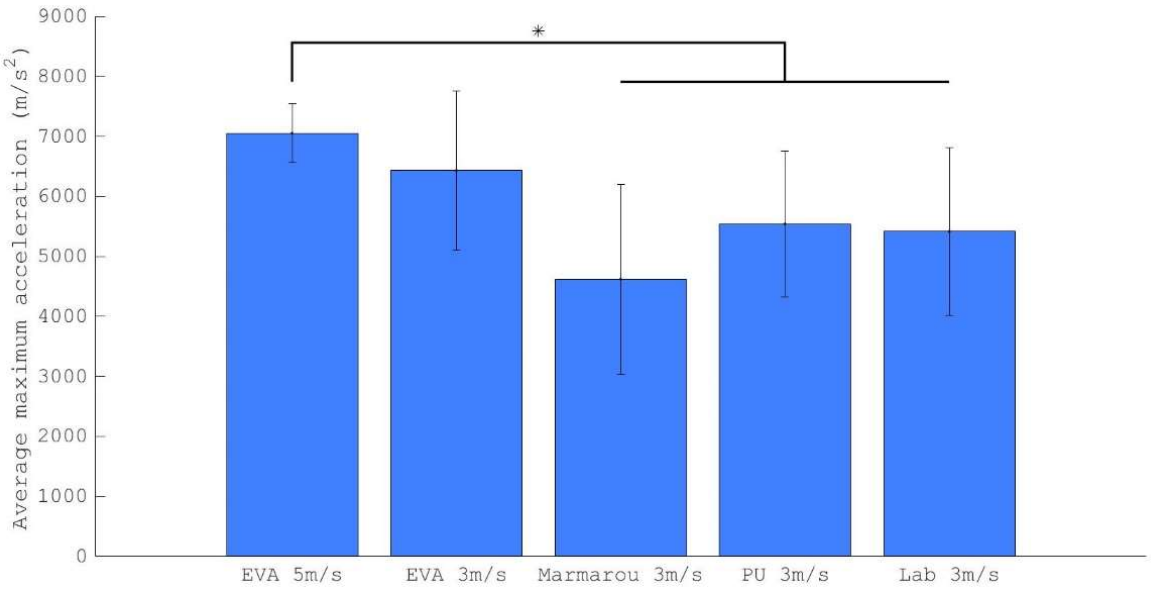

Figure 4.5 Average peak acceleration of the head following single impact

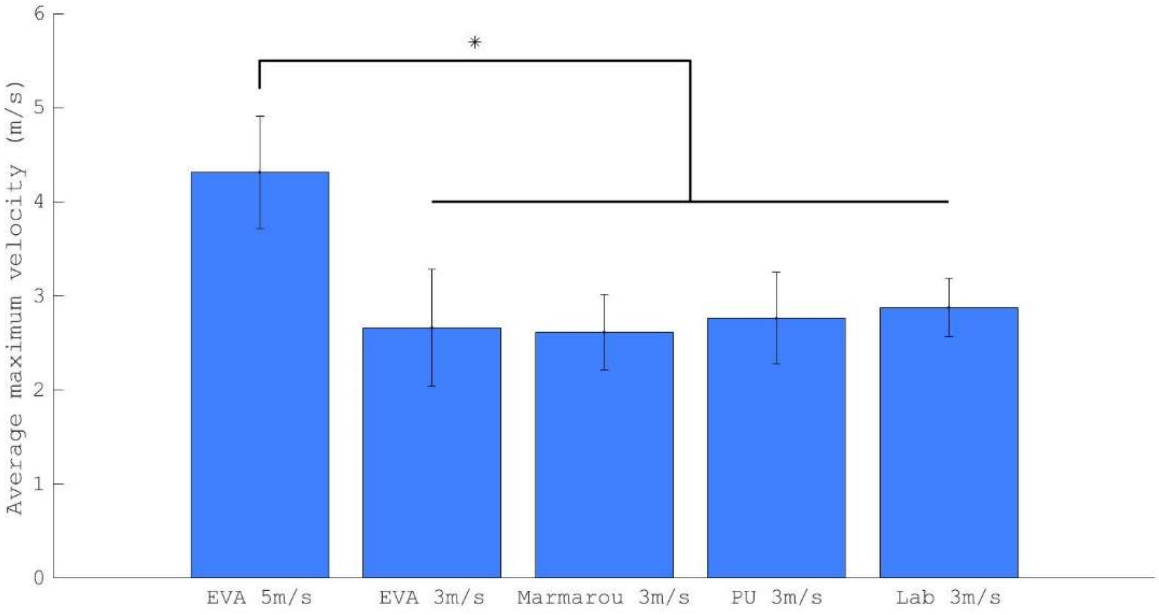

Figure 4.6 Average peak velocity of the head following single impact

Table 4.2 R^2 values from regression on foam properties and biomechanical variables against density and Young's modulus

	Density (g/cm^3)	Young's modulus (kPa)
Peak acceleration (m/s^2)	$R^2 = 0.12$	$R^2 = 0.08$
Average acceleration (m/s^2)	$R^2 = 0.33$	$R^2 = 0.28$
Peak velocity (m/s)	$R^2 = 0.12$	$R^2 = 0.09$
Average velocity (m/s)	$R^2 = 0.14$	$R^2 = 0.09$

4.2 Acute neurological response

Toe pinch response times were not significantly affected by anesthesia duration, rat weight, or the type of foam used during injury, and were not significantly different from shams. Righting reflex times were increased by prolonged anesthesia duration ($p < 0.05$), but were not significantly affected by animal weight or the type of foam used during injury. Mean neurological responses are given in Table 4.3.

Table 4.3 Average acute neurological response to TBI

	Sham	EVA	PU	Marmarou	Lab
Anesthesia duration (s)	1019±231	1144±298	1018±189	969±25	1157±261
Toe pinch response (s)	172±150	268±145	189±147	88±64	188±100
Righting reflex (s)	223±113	222±171	125±120	223±52	127±88

4.3 Tissue response

4.3.1 Representative slides

Nine images were taken of the cortex of male rats. Three sections were selected from the anterior, central, and posterior aspects of the injury site, and three images were then taken from each section: one from each hemisphere and one from near the midline of the brain. Representative images are given in Figures 4.7 – 4.11.

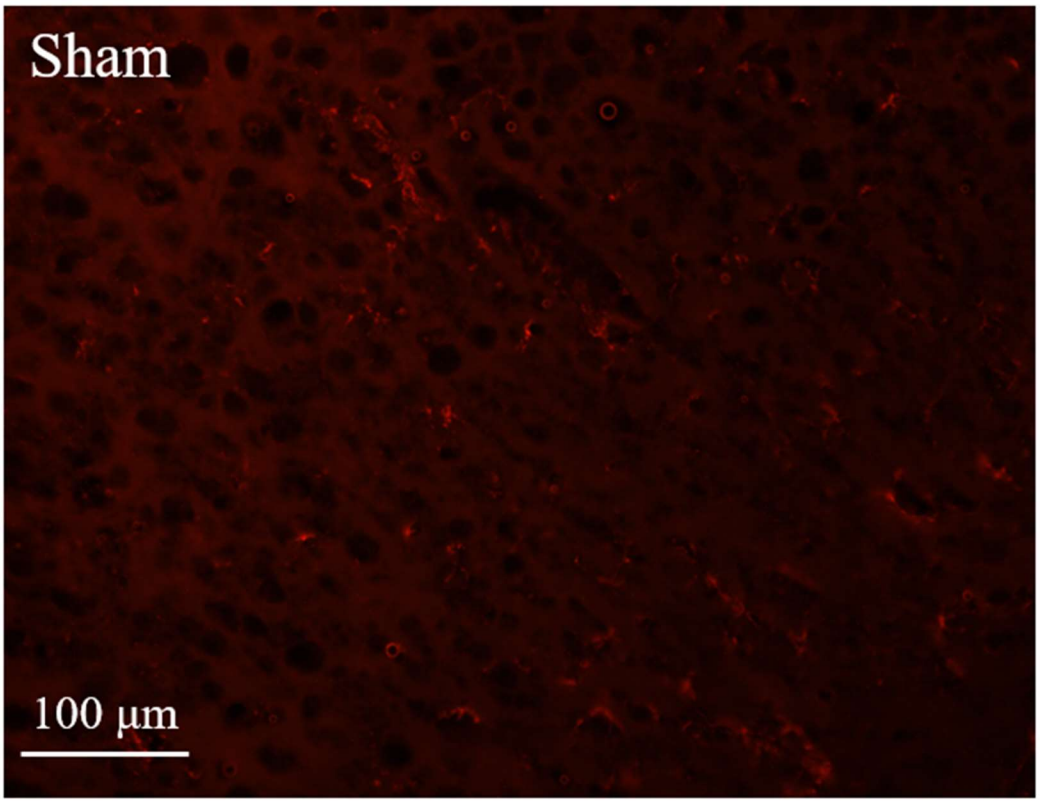

Figure 4.7 Image from uninjured sham rat

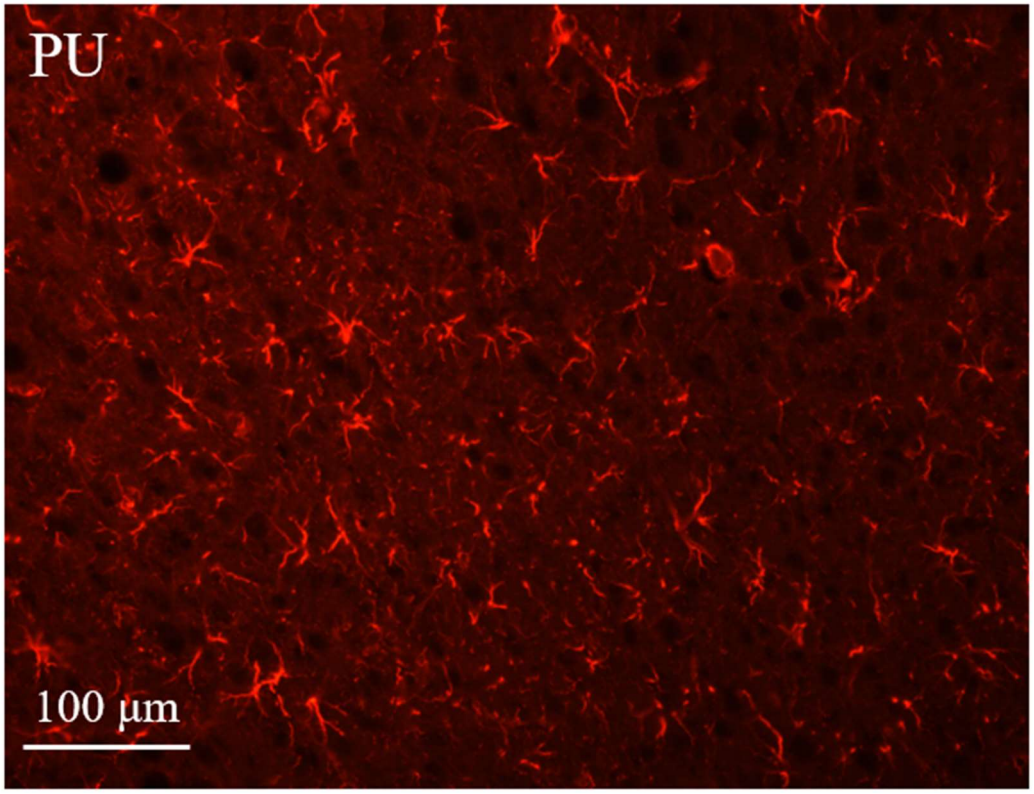

Figure 4.8 Image from rat injured on PU foam, 3 m/s

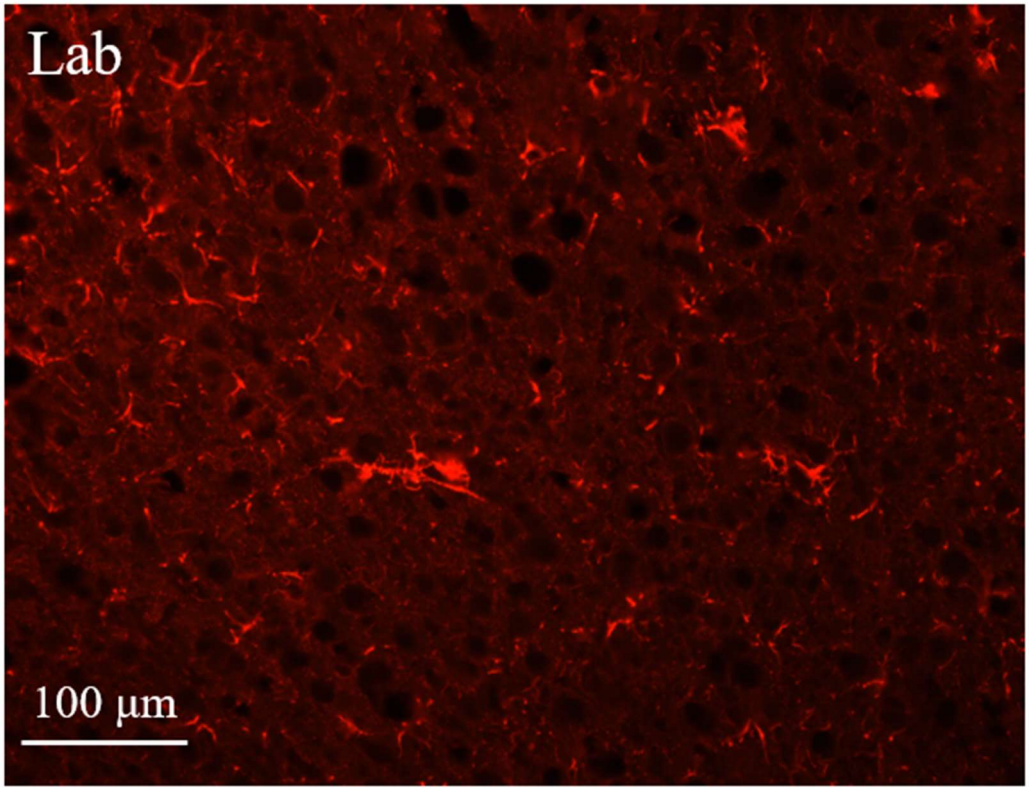

Figure 4.9 Image from rat injured on Lab foam, 3 m/s

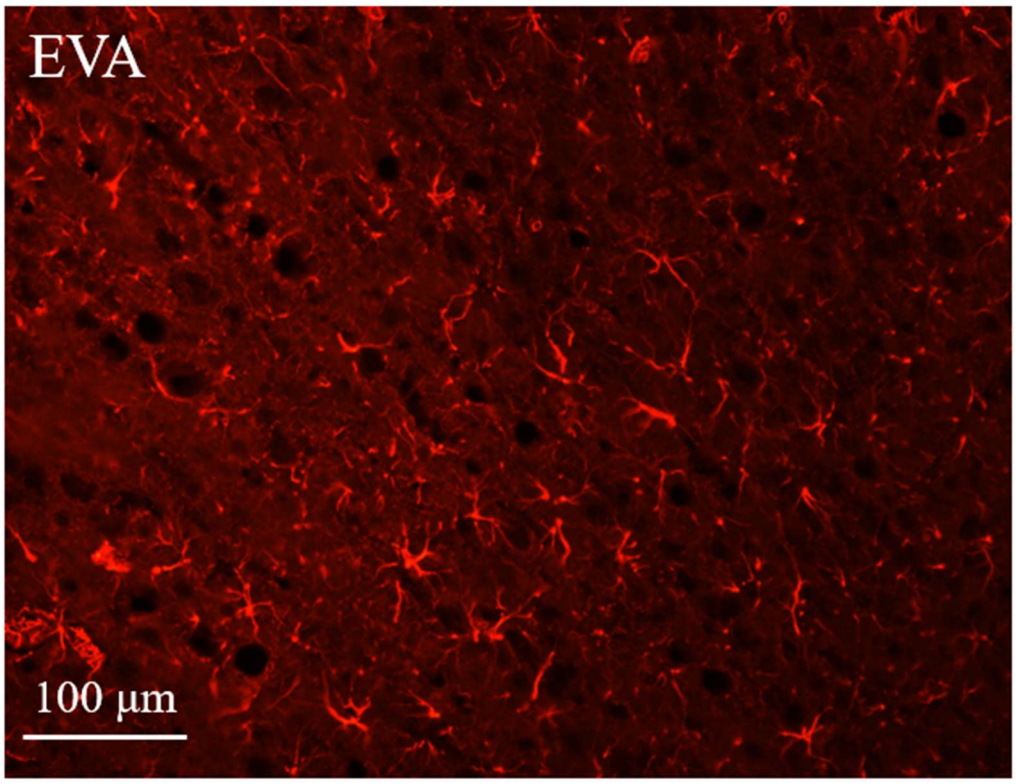

Figure 4.10 Image from rat injured on EVA foam, 3 m/s

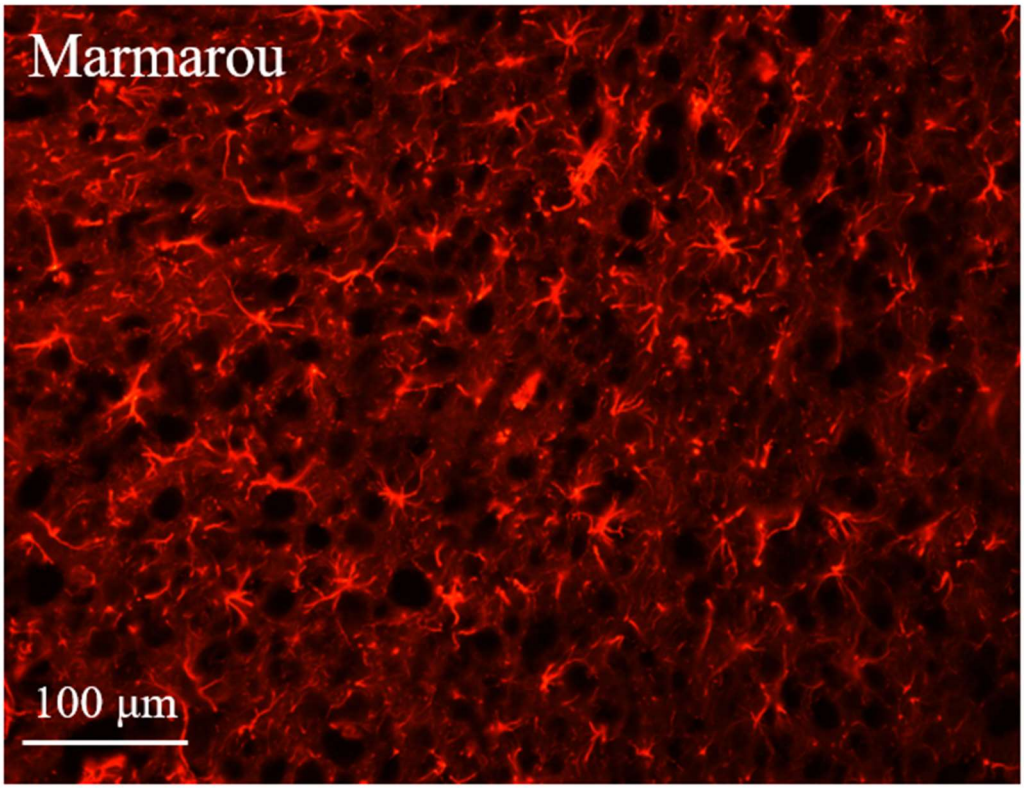

Figure 4.11 Image from rat injured on Marmarou foam, 3 m/s

4.3.2 Foam effects on GFAP expression

Images were processed using a free distribution of ImageJ (NIH). Images were first preprocessed by applying a binary mask based on pixel intensity, using ImageJ default "Dark" settings to account for background noise and separate areas of GFAP expression. An ImageJ macro was then used to count the number of separate GFAP-expressing astrocytes (see Appendix). The same thresholding and counting macro was used to process all images. Mean cell counts were calculated for each foam under the 3m/s injury condition (Figure 4.12).

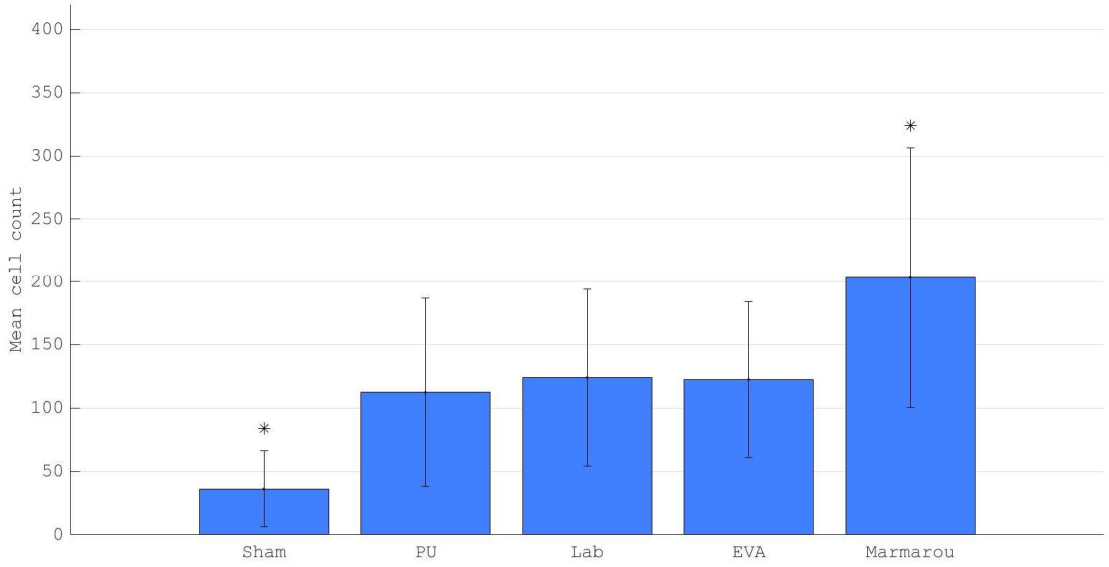

Figure 4.12 Mean number of GFAP expressing cells for each foam type

Injured brains all expressed significantly elevated levels of GFAP compared to sham brains, regardless of foam type ($p < 0.05$). GFAP expression in brains injured on Marmarou foam was higher than in brains injured on EVA, PU, or Lab

foam ($p < 0.05$). There was no significant difference in GFAP expression between brains injured on PU foam, EVA foam, or Lab foam. However, due to low sample numbers in the Marmarou group, these GFAP findings are inconclusive. Individual animals that experienced rebound impacts did not exhibit significantly more GFAP expression than those that did not, for all foam types. R^2 values for linear regression on foam density and Young's modulus against GFAP expression were 0.04 and 0.006, respectively.

CHAPTER 5

DISCUSSION

5.1 Foam selection affects injury biomechanics

We found that foam selection had two main effects on injury biomechanics. First, foams with a lower Young's modulus permitted the head a greater range of motion. This was expected, since Young's modulus describes material stiffness, and a more pliable material should allow more deformation under the same load.

Secondly, rebound impacts were observed on all foams, although rebound differences between foams did not reach statistical significance. Rebound impacts have been observed previously in weight drop models of TBI (Xiong et al. 2013), but to our knowledge this phenomenon has not been reported in previous literature on CCI models of TBI. Rebound hits are not readily detectable with the naked eye; high-speed video of the moment of impact is required.

We did not find a significant difference in tissue response between rebounded brains and those who were only impacted once. However, the presence of these rebounds in some injuries is a potential source of error and merits further study. The inconsistency of rebounds is also of note, and is potentially due to experimental error. The exact positioning of the head on the foam block, and the precise location of impact are likely to play a role in the presence or absence of rebounds. It is also possible that movement of

the head due to breathing plays a role in the differences we observed. Finally, it is possible that not all rebounds are the same; although our results did not show that rebound impacts affected injury response, the severity of the rebound impact may vary between foams.

5.2 Foam selection affects tissue response following TBI

We found that foam selection may affect GFAP expression in injured brains. GFAP was expressed in significant amounts in all injured brains, compared to uninjured shams, but was most highly expressed in brains injured on Marmarou foam. However, low n prevents this finding from reaching statistical significance. Although they had divergent material properties, PU, EVA, and Lab foams generated comparable levels of GFAP expression. However, the GFAP levels exhibited in brains injured on Lab foam and Marmarou foam were different, despite those foams' material similarities. We found that foam density and Young's modulus were not predictive of injury outcome. This suggests that the relationship between material properties and GFAP expression is more complex than a simple function of Young's modulus or foam density.

Furthermore, since the total range of motion correlated well with Young's modulus but the tissue response did not,

biomechanics alone was not enough to predict tissue response. It is likely that a combination of factors, including experimental error and individual animal variability, contributed to the disparate relationships between foam type, biomechanics, and tissue response. Note that we did not pair accelerometer measurements with GFAP counts; further work and increased sample size would permit analysis of the connection between acceleration, velocity, and tissue response. It is also possible that our injury model is too mild to generate deficits strong enough to overcome the level of noise in our measurements.

5.3 Foam properties and biomechanics do not predict injury outcome

Despite the increase in GFAP expression following injury, our results show that these effects are not captured by the material properties of the foams or the biomechanical measurements alone. For this reason, we recommend that the selection of head support material be approached similarly to other TBI model design parameters, such as the type of injury model or whether the head is fixed or unfixed. That is, the type of foam and its supplier should be reported and included as a potential confounder between models, but extensive

material and/or biomechanical characterization of individual foam selections is likely unnecessary.

Furthermore, we expect that the variety of materials used to support the head may contribute to model heterogeneity. As has been reported previously (Voelkl and Würbel 2016, Voelkl et al. 2018), this may be desirable if carefully implemented and reported. In our case, we have elected to use the EVA foam from McMaster-Carr in future injury models because it exhibited fewer rebound impacts and its range of motion was closest to what was prescribed.

In addition to head support material, there are a number of other unreported features that are involved in preclinical TBI models. These are not limited to variables that describe the injury model itself, but include descriptions of the animals and of the outcome measures as well. We are currently analyzing the effects of population heterogeneity on TBI outcomes, and are collaborating on a common data elements framework that will permit more thorough and consistent reporting of these potentially confounding variables. Preliminary results of this work are presented and discussed in Chapter 6.

CHAPTER 6

ONGOING WORK

6.1 Population heterogeneity & TBI

6.1.1 Introduction

Preclinical models of TBI are generally designed to be as homogeneous as possible, in order to eliminate confounding variables. This approach, however, is unable to capture the heterogeneity of the clinical TBI population, limiting the ability to validate animal models as research surrogates. The female sex hormones estrogen and progesterone, for example, have been demonstrated to have a neuroprotective effect in rats following TBI or stroke (Stein 2001). Similarly, animal strains have been shown to respond differently to injury. In one study, Sprague Dawley rats recorded fewer seizures and had less severe histopathological injury response than Fischer rats following fluid percussion injury, although Fischer rats performed better on cognitive tests than their Sprague Dawley counterparts (Reid et al. 2010).

6.1.2 Study design

In this study, we introduce a preclinical model of population heterogeneity in TBI. Namely, we separate a population of 144 young adult (75-90 days old) rats into

groups consisting of combinations of both sexes, two strains (Sprague Dawley and Fischer), three injury types, and three sacrifice times (n=4 per group). We then administer 0, 1, or 2 mild traumatic brain injuries according to the protocol described in Chapter 3, at 5m/s with 5mm displacement and a contact time of 50ms. EVA foam is used to support the head. Repeat injuries are administered 24h apart. Behavioral effects are evaluated 4h post-injury and again just prior to sacrifice, using a novel object recognition (NOR) task and a gait assay. Each animal is sacrificed either 24h, 72h, or 1 week after their final injury and histopathological methods are used to evaluate tissue response. In this chapter, we present preliminary results from the NOR task.

6.1.3 NOR methods

Novel object recognition (NOR) is a common behavioral task used to evaluate short-term memory in rodents. Previous rodent studies have shown that for visual object recognition memory, the parahippocampal regions of the temporal lobe are important, in particular the perirhinal, entorhinal, and inferior temporal cortices. NOR deficits may indicate damage to these areas (Hammond et al. 2004). A number of variations on the basic NOR procedure have been reported; our method was based on a common 2-object technique (Antunes and Biala 2012).

For this test, the animal was first habituated to the NOR container, a 2’x2’x2’ grey plastic box, for 10 minutes the day prior to testing. The day of testing, the animal was given a 5 minute refresher in the box, before being removed and returned to the home cage. Two similar but unidentical objects, Object 1 and Object 2, were placed in opposite and symmetrical corners of the test box. The animal was reintroduced to the box for 3 minutes; this is the *acquisition phase*. The time spent exploring each object was recorded. Exploring was defined as time the animal spent within 5cm of the object, facing it, sniffing it, and/or touching it. Incidental contact is not considered. The animal was again removed and returned to the home cage for 15 minutes, during which time the box was wiped clean of any debris and Object 2 replaced with Object N, a novel object. The animal was placed back in the box for 3 minutes, the *retention phase*, and the time spent exploring each object was recorded.

The animal was always placed in the center of the box, facing the same direction, and objects were always placed in the same two corners of the box. In this study, we used three glass bottles for Objects 1, 2, and N. Objects 1 and 2 were clear glass with a black or yellow plastic cap and had a similar silhouette, while Object N was a larger bottle made

of brown glass with a black plastic cap. The box and objects were wiped down with 10% ethanol following each test.

A total of four time measurements were recorded for each test: the time spent exploring Object 1 or Object 2 during the acquisition phase (A1, A2), and the time spent exploring Object 1 or Object N during the retention phase (R1, RN). To control for individual differences in exploration predilection, these measurements were combined to create two index variables, Retention Index and Acquisition-Retention Difference (Antunes and Biala 2012). Retention index is the percent time spent exploring the novel object during the retention phase, defined as R_{INDEX} = RN/(R1 + RN). Acquisition-Retention Difference is defined as $AR_{DIFF} = R_{INDEX} - A_{INDEX}$, where A_{INDEX} = A2/(A1 + A2). AR_{DIFF} was used as the primary outcome measure for this study.

6.1.4 Preliminary NOR results

Preliminary results show that short-term memory was impaired in injured animals compared to shams for at least 24 hours, regardless of sex or strain ($p < 0.05$). Animals that received two injuries displayed more severe deficits than those that received just one, for at least 24 hours after injury ($p < 0.05$), but not at later timepoints (Figures 6.1, 6.2).

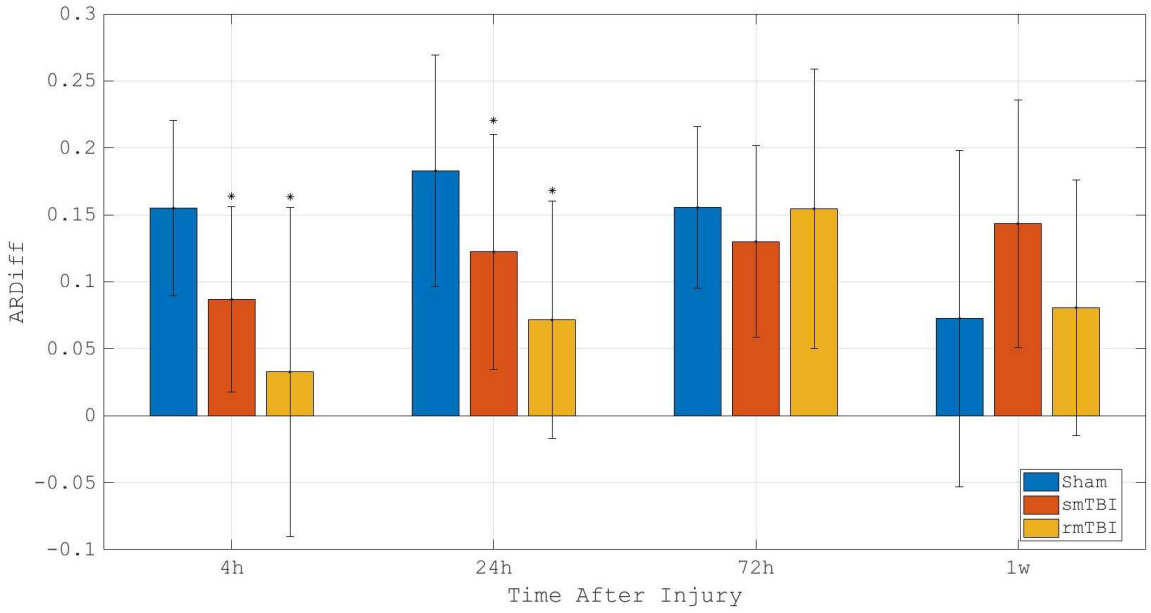

Figure 6.1 Overall effects on NOR performance following single (sm) or repeat (rm) TBI compared to shams

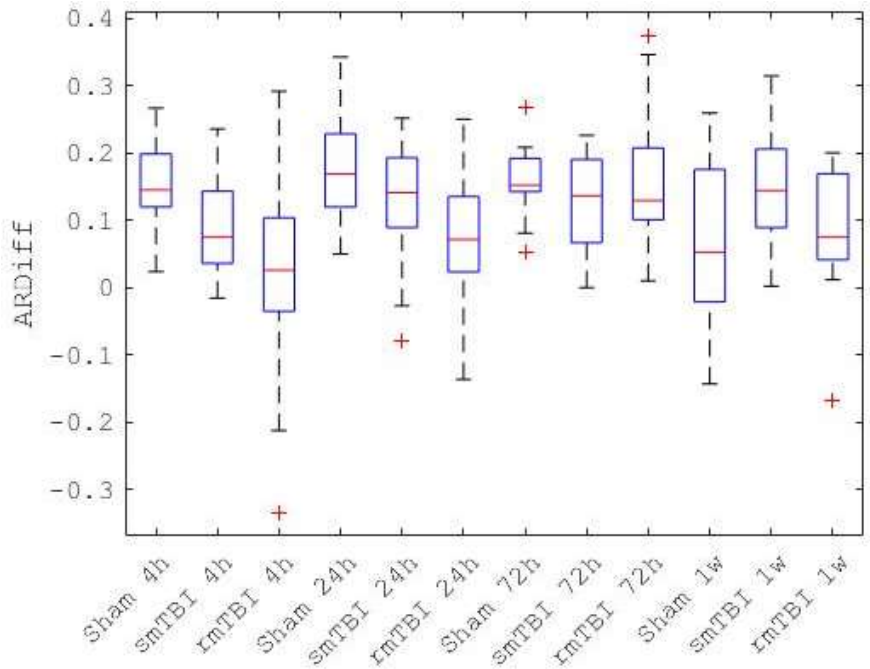

Figure 6.2 Boxplot of overall NOR performance following sham, single (sm), or repeat (rm) TBI

Female rats that received either one or two injuries, and male rats that received two injuries, were significantly impaired for at least 24 hours after injury ($p < 0.05$). Male

rats that received only one injury had recovered by 24h and their performance was indistinguishable from shams (Figures 6.3, 6.4).

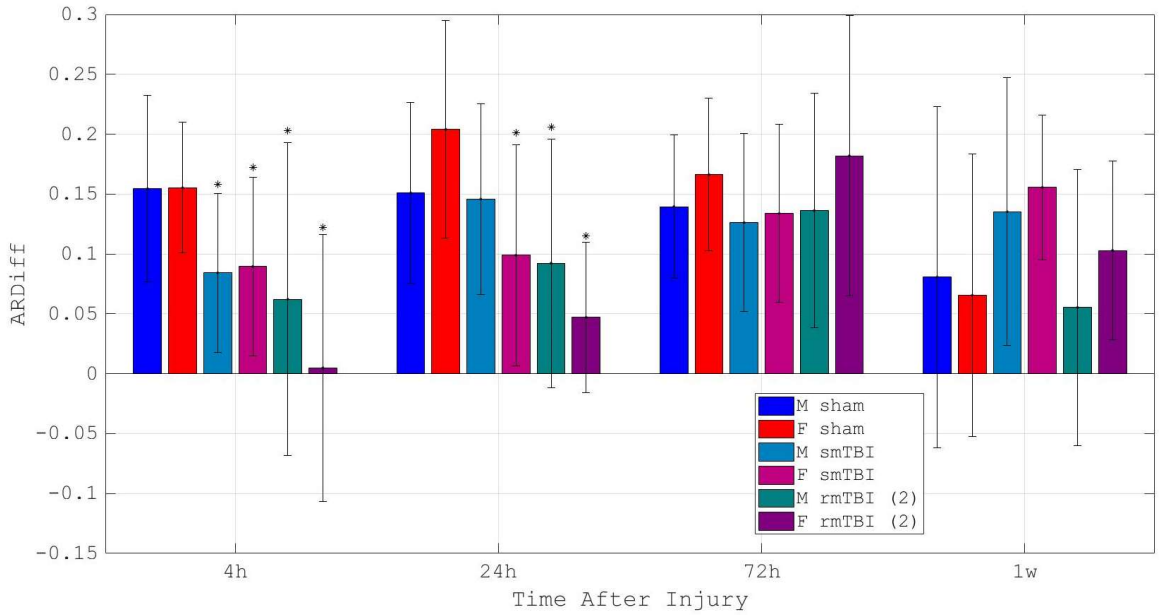

Figure 6.3 Sex effects on NOR performance following single (sm) or repeat (rm) TBI compared to shams

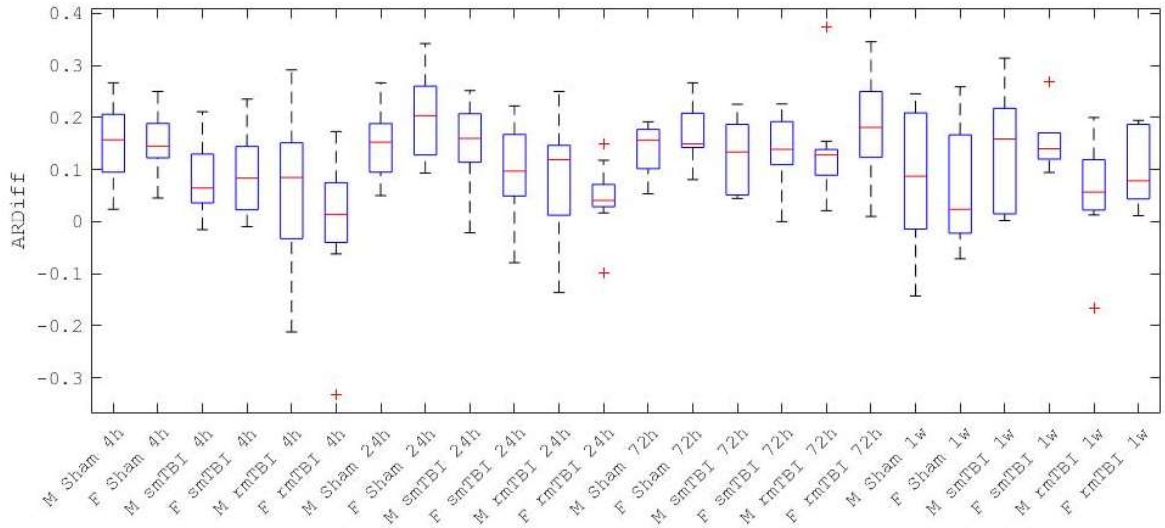

Figure 6.4 Boxplot of sex effects on NOR performance following sham, single (sm), or repeat (rm) TBI

Preliminary results also show that animal strain may affect NOR performance following TBI. Both Sprague Dawley (SD) and Fischer (FS) strains presented with deficits 4 hours after injury, but only Sprague Dawley animals that were injured twice still showed significant deficits after 24 hours ($p < 0.05$) (Figures 6.5, 6.6). All animals had recovered by 72 hours.

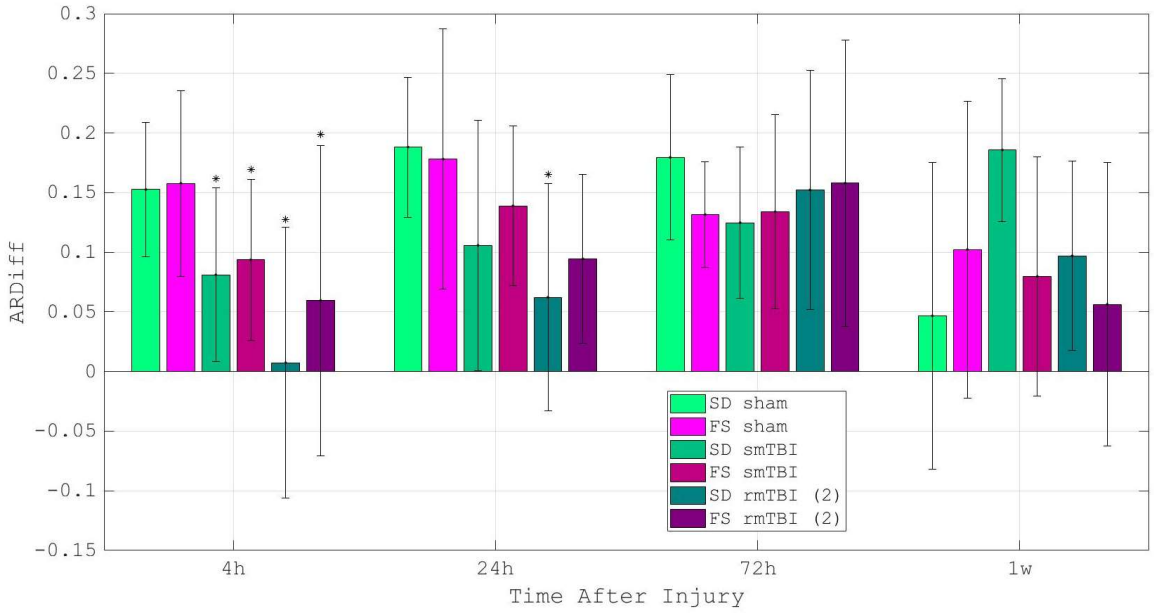

Figure 6.5 Strain effects on NOR performance following single (sm) or repeat (rm) TBI compared to shams

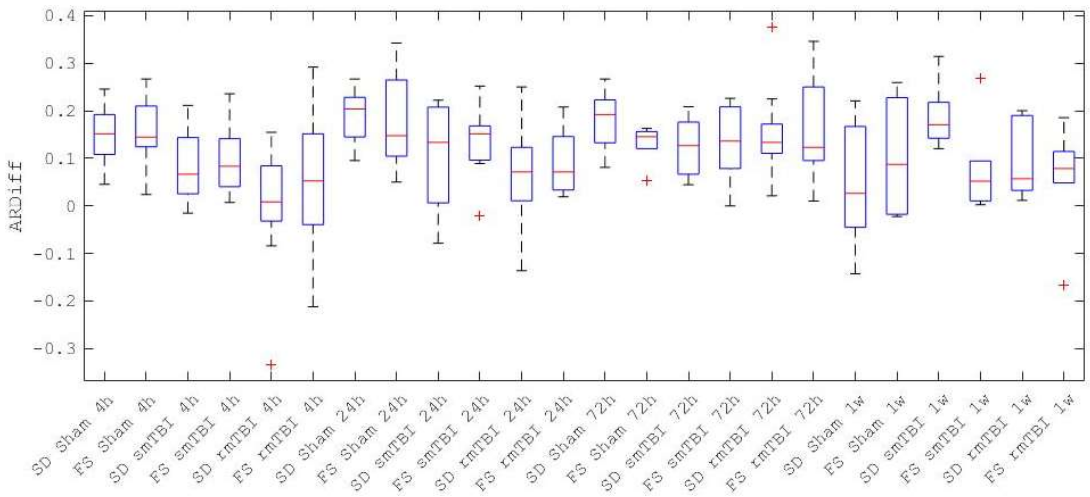

Figure 6.6 Boxplot of strain effects on NOR performance following sham, single (sm), or repeat (rm) TBI

6.1.5 Discussion

Sex and strain effects on TBI outcome have been noted previously in literature, and our preliminary NOR results also show sex- and strain-based differences. We found that female rats recovered more slowly than males of the same age who underwent a comparable injury. This is contrary to some prior evidence that shows a neuroprotective effect of female sex hormones (Stein 2001). However, we did not match the estrous cycles of female subjects so hormonal levels were likely variable between individuals. We also found that Sprague Dawley rats displayed more severe memory deficits and recovered more slowly than Fischer rats. This is in line with previous work showing that, although Fischer rats show more tissue damage and display greater motor deficits, they perform better on cognitive tasks following brain injury (Reid et al. 2010).

We are currently working on a statistical model to analyze the interactions between sex, strain, weight, time, and the number of injuries received. Forthcoming data include gait analysis (Noldus Catwalk XT) and histopathological results. Additionally, we are evaluating the effects of 3 and 5 repeated mild injuries on gait and balance.

6.2 Preclinical common data elements

In recent years, there has been a significant effort within the clinical TBI research community to improve reporting and develop more precise outcome measures by developing a set of Common Data Elements (CDEs), to be used among labs via a shared data repository (Yue et al. 2013). CDEs have recently started to find use in preclinical research, with the aims of improving reproducibility and rigor by standardizing reporting without standardizing experimental methods.

These aims are in line with our own work on preclinical model and population heterogeneity, the results of which demonstrate that more thorough and standardized reporting of experimental variables is necessary. We are currently collaborating with several other research groups, as well as the NIH, to develop a set of CDEs that will be able to more fully quantify and describe preclinical experimental methods

and outcomes. The working set of preclinical CDEs is available at https://fitbir.nih.gov/content/preclinical-common-data-elements.

CHAPTER 7

CONCLUSIONS

In this study, we evaluated the biomechanical and tissue response differences of using various foams to support the head during a closed-head CCI injury in rats. We found that such differences exist but are not captured by basic material properties, like density or Young's modulus, or by biomechanical differences between foams. This indicates that more consistent reporting of foam selection is desirable for reproducibility, but that material characterization alone is inadequate to address the effects of foam selection.

In conclusion, we recommend further investigation into the role that other model design variables may play in unintended heterogeneity. We acknowledge that planned, controlled heterogeneity is beneficial but that under-reported differences between protocols may be confounders. We therefore suggest that material type, size, and supplier should be consistently reported in TBI models that support the head, but advanced material analysis and characterization is likely unnecessary. We also recommend moving towards more comprehensive reporting of other model design choices. To this end, we support the adoption of preclinical common data elements as a reporting tool.

APPENDIX

ImageJ thresholding & cell counting code:

```
input = getDirectory("D:\Research");
output = getDirectory("D:\Research");
suffix = ".tif";
setBatchMode(true);
processFolder(input);
function processFolder(input) {
     list = getFileList(input);
     for (i = 0; i < list.length; i++) {
          if(endsWith(list[i], suffix))
               processFile(input, output, list[i]);
          }
}
function processFile(input, output, file) {
     print("Processing: " + input + file);
     open(input + file);
     run("16-bit");
     setAutoThreshold("Default dark");
     //run("Threshold...");
     setThreshold(65, 200);
     setOption("BlackBackground", true);
     run("Convert to Mask");
     saveAs("Tiff", output + file + "_BW.tif");
     run("Analyze      Particles...",      "size=25-Infinity
circularity=0.00-0.60    show=Outlines    display    clear
summarize");
     saveAs("Jpeg", output + file + "_Drawing.jpg");
     close();
     saveAs("Results", output + file + "_Summary.csv");
     close();
     saveAs("Results", output + file + "_Results.csv");
     run("Close");
}
```

REFERENCES

Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cognitive Processing. 2012; 13:93-110.

Bramlett HM, Dietrich WD, Green EJ. Secondary hypoxia following moderate fluid percussion brain injury in rats exacerbates sensorimotor and cognitive deficits. J Neurotrauma. 1999; 16:1035-1047.

Carbonell WS, Maris DO, McCall T, Grady MS. Adaptation of the fluid percussion injury model to the mouse. J Neurotrauma. 1998; 15:217-229.

Carroll L, et al. Methodological issues and research recommendations for mild traumatic brain injury: the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury. J Rehabil Med. 2004; 43:113-125.

Chen Y, Constantini S, Trembovler V, Weinstock M, Shohami E. An experimental model of closed head injury in mice: pathophysiology, histopathology, and cognitive deficits. J Neurotrauma. 1996; 13:557-568.

Cheng J, et al. Development of a rat model for studying blast-induced traumatic brain injury. J Neurol Sci. 2010; 294:23-28.

Cikriklar HI, et al. Effectiveness of GFAP in Determining Neuronal Damage in Rats with Induced Head Trauma. Turk Neurosurg. 2016; 26: 878-889.

Feeney DM, Boyeson MG, Linn RT, Murray HM, Dail WG. Responses to cortical injury: I. Methodology and local effects of contusions in the rat. Brain Res. 1981; 211:67-77.

Flierl MA, et al. Mouse closed head injury model induced by a weight-drop device. Nat Protoc. 2009; 4:1328-1337.

Furger RE, Nelson LD, Lerner EB, McCrea MA. Frequency of factors that complicate the identification of mild traumatic brain injury in level I trauma center patients. Concussion. 2015; 1(2).

Galgano M, et al. A Review of Traumatic Brain Injury Animal Models: Are We Lacking Adequate Models Replicating Chronic Traumatic Encephalopathy? J Neurol Neurobiol. 2015; 2(1). DOI: http://dx.doi.org/10.16966/2379-7150.117

Grayson B, et al. Assessment of disease-related cognitive impairments using the novel object recognition (NOR) task in rodents. Behavioral Brain Research. 2015; 285:176-193.

Hamm RJ, et al. Cognitive Deficits Following Traumatic Brain Injury Produced by Controlled Cortical Impact. J Neurotrauma. 1992; 9:11-20.
Hammond RS, Tull LE, Stackman RW. On the delay dependent involvement of the hippocampus in object recognition memory. Neurobiol Learn Mem. 2004; 82:26–34.
Jamnia N, et al. A Clinically Relevant Closed-Head Model of Single and Repeat Concussive Injury in the Adult Rat Using a Controlled Cortical Impact Device. J Neurotrauma. 2016; 33:1-13.
Johnson VE, Meaney DF, Cullen DK, Smith DH. Animal models of traumatic brain injury. Handb Clin Neurol. 2015; 127: 115-128.
Johnson VE, Stewart W, Smith D. Traumatic brain injury and amyloid-β pathology: a link to Alzheimer's disease? Nat Rev Neurosci. 2010; 11(5):361-370.
Kilbourne M, et al. Novel model of frontal impact closed head injury in the rat. J Neurotrauma. 2009; 26:2233–2243.
Lighthall J. Controlled Cortical Impact: A New Experimental Brain Injury Model. J Neurotrauma. 1988; 5:1-15.
Long JB, et al. Blast overpressure in rats: recreating a battlefield injury in the laboratory. J Neurotrauma. 2009; 26:827–840.
Marmarou A, et al. A new model of diffuse brain injury in rats. J Neurosurg. 1994; 80(2):291-300.
McIntosh TK, Noble L, Andrews B, Faden AI. Traumatic brain injury in the rat: characterization of a midline fluid-percussion model. Cent Nerv Syst Trauma. 1987; 4:119–134.
Mychasiuk R, Farran A, Angoa-Perez M, Briggs D, Kuhn D, Esser MJ. A Novel Model of Mild Traumatic Brain Injury for Juvenile Rats. J Vis Exp. 2014; (94), e51820, DOI:10.3791/51820
National Institutes of Health (NIH). Traumatic brain injury: Hope through research. NIH Publication No. 02-2478. 2002.
Papa L, et al. GFAP Out-Performs S100β in Detecting Traumatic Intracranial Lesions on Computed Tomography in Trauma Patients with Mild Traumatic Brain Injury and Those with Extracranial Lesions. J Neurotrauma. 2014; 31:1815-1822.
Petraglia AL, Plog BA, Dayawansa S, Dashnaw ML, Czerniecka K, Walker CT, et al. The pathophysiology underlying repetitive mild traumatic brain injury in a novel mouse model of chronic traumatic encephalopathy. Surg Neurol Int 2014; 5:184.

Prins ML, Hales A, Reger M, Giza CC, Hovda DA. Repeat Traumatic Brain Injury in the Juvenile Rat is Associated with Increased Axonal Injury and Cognitive Impairments. Dev Neurosci. 2010; 32:510-518.

Rachmany L, Tweedie D, Rubovitch V, Yu Q-S, Li Y, et al. Cognitive Impairments Accompanying Rodent Mild Traumatic Brain Injury Involve p53-Dependent Neuronal Cell Death and Are Ameliorated by the Tetrahydrobenzothiazole PFT-α. PLoS ONE. 2013; 8(11): e79837.

Reid WM, Rolfe A, Register D, Levasseur JE, Churn SB, Sun D. Strain-Related Differences after Experimental Traumatic Brain Injury in Rats. J Neurotrauma. 2010; 27:1243-1253.

Reneer DV, et al. A multi-mode shock tube for investigation of blast-induced traumatic brain injury. J Neurotrauma. 2011; 28:95–104.

Siopi E, et al. Evaluation of late cognitive impairment and anxiety states following traumatic brain injury in mice: the effect of minocycline. Neurosci Lett. 2012; 511(2):110-115.

Smith D, et al. Pre-Clinical Traumatic Brain Injury Common Data Elements: Toward a Common Language Across Laboratories. J Neurotrauma. 2015; 32:1725-1735.

Stein D. Brain damage, sex hormones and recovery: a new role for progesterone and estrogen? Trends in Neurosciences. 2001; 24:386-391.

Taylor CA, Bell JM, Breiding MJ, Xu L. Traumatic Brain Injury–Related Emergency Department Visits, Hospitalizations, and Deaths — United States, 2007 and 2013. MMWR Surveill Summ 2017; 66(No. SS-9):1–16. DOI: http://dx.doi.org/10.15585/mmwr.ss6609a1

Voelkl B, Würbel H. Reproducibility Crisis: Are We Ignoring Reaction Norms? Trends in Pharm Sci. 2016; 37(7): 509-510.

Voelkl B, Vogt L, Sena ES, Würbel H. Reproducibility of preclinical animal research improves with heterogeneity of study samples. PLoS Biol. 2018; 16(2): e2003693. https://doi.org/10.1371/journal.pbio.2003693.

Wojnarowicz MW, Fisher AM, Minaeva O, Goldstein LE. Considerations for Experimental Animal Models of Concussion, Traumatic Brain Injury, and Chronic Traumatic Encephalopathy— These Matters Matter. Front. Neurol. 2017; 8:240

Xiong Y, Mahmood A, Chopp M. Animal models of traumatic brain injury. Nat Rev Neurosci. 2013; 14:128-142.
Yue J, et al. Transforming Research and Clinical Knowledge in Traumatic Brain Injury Pilot: Multicenter Implementation of the Common Data Elements for Traumatic Brain Injury. J Neurotrauma. 2013; 30:1831-1844.
Zhang S, Wu M, Peng C, Zhao G, Gu R. GFAP expression in injured astrocytes in rats. Experimental and Therapeutic Medicine. 2016; 14:1905-1908.